Appendix 6

Appendix 6

Research Protocol 936-9216

"Bactericidal Efficacy of Essential Oil-Containing Mouthrinses Against Oral Microorganisms *in vitro*"

Protocol for Warner-Lambert Co.
Consumer HealthCare Research & Development
Oral Care

Bactericidal Efficacy of Essential Oil-Containing Mouthrinses Against Oral Microorganisms *in vitro*

Study #936-9216
May 10, 2000

Principal Investigator:

Michael Rubin
Senior Scientist
Oral Care

_____ Signature _____ Date

Warner-Lambert Monitor

Pauline Pan, Ph.D.
Research Fellow
Oral Care

_____ Signature _____ Date

Warner-Lambert Statistician

James A. McGuire
Senior Res. Assoc.
Statistics & Data Management

_____ Signature _____ Date

1 **Objective**

To determine the comparability of the bactericidal activity of a Listerine Antiseptic mouthrinse formulation with 0.02% sodium fluoride with that of Listerine Antiseptic.

2 **Experimental Design:**

2.1 This kill kinetics study is based on a protocol accepted by the FDA Plaque Products Subcommittee as one of two performance tests required to ensure that the fixed ratio of four essential oils contained in Listerine Antiseptic mouthrinse retains comparable activity in a new formulation. This study will compare the rapid bactericidal action of a fluoride-containing Listerine formulation (FreshBurst Listerine Antiseptic with 0.02% sodium fluoride) to that of a currently marketed Listerine mouthrinse (Listerine Antiseptic). The effectiveness of the two products in killing laboratory grown and wild-type (salivary) microorganisms during a thirty second exposure will be determined.

2.2 **Random code**: The products will be tested according to a randomization schedule provided by the Statistics and Data Management Department. Within each test day, products will be run in a random order.

3 **Test Materials:**

3.1 The test formulation consists of FreshBurst Listerine Antiseptic to which 0.02% sodium fluoride has been added. The positive and negative control formulations are Listerine Antiseptic and a 5% hydroalcoholic solution, respectively.

PRODUCT NAME	PURPOSE	W/LH #
FreshBurst Listerine with 0.02% sodium fluoride	Test Product	W2194-471
Listerine Antiseptic	Positive Control	W2194-377
5% hydroalcoholic negative control	Negative Control	W2194-221P

4 **Media:**

All organisms will be grown in Schaedler broth (Difco) and maintained on Schaedler agar (Difco) supplemented with 5% sheep's blood. Dilutions will be made with 0.1% peptone (Difco) blanks (9.9 mL). Schaedler broth (Difco) supplemented with azolectin and Tween 80 will be used to stop the reaction and is referred to as neutralizing broth

4.1 Neutralizing Broth

Neutralizing broth is comprised of Schaedler broth supplemented with Azolectin and Tween 80 (Warner Lambert Research Report #952-0137).

Dissolve 28.4 grams of Schaedler broth in 900 mL of distilled water. Add 15 mL of Neutralizing Solution Stock. q.s. to 1 liter with distilled water, and dispense into tubes (4.95 mL/tube), and autoclave.

4.2 Neutralizing Solution Stock

Combine 35 grams of Azolectin (L-Phosphatidylcholine) with 700 mL of distilled water. Heat and mix until Azolectin is dissolved. Add 250 grams of Tween 80 (Polysorbate 80 NF) and 1 mL of potassium phosphate buffer. Heat and mix until dissolution is complete. Stock will become clear. q.s. to 1 liter with water. Cool to ambient temperature and adjust pH to 7.2 with 1N NaOH. Autoclave in a glass container. Solution should be stored refrigerated (4°C).

4.3 Potassium Phosphate Buffer

Dissolve 3.74 grams of KH_2PO_4 in 90 mL of distilled water. Adjust pH to 7.2 with 1N NaOH and q.s. to 100 mL with distilled water.

Neutralizing Test

This test is conducted simultaneously with the product testing for each test organism to assure adequate product neutralization thus permitting organism growth. Add 0.1 mL of test solution to 10 mL of neutralizing broth. Prepare test culture as for a standard test. Prepare 1:100 dilution of each culture by serial dilution in growth media. Within 15 minutes of making the 1:100 culture dilution, inoculate the diluted test solution with 0.1 mL of the 1:100 culture. Incubate inoculated tubes. Visible growth within 48 hours of inoculation indicates effective neutralization.

5 Organisms:

The following ATCC registered strains will be used for testing:

- *Fusobacterium nucleatum* (ATCC #10953)
- *Prevotella intermedia* (ATCC #25611)
- *Actinomyces viscosus* (ATCC #19246)
- *Streptococcus sanguis* (ATCC #10556)
- *Streptococcus mutans* (ATCC #25175)
- *Candida albicans* (ATCC #18804)

5.1 The organisms will be grown anaerobically at approximately 37°C in a Coy anaerobic chamber with an atmosphere of 2.0> H_2 <5.0 % , 5% CO_2, balance N_2, with the exception of *C. albicans*, which will be cultivated aerobically at approximately 30° C.

5.2 The inoculum for the test will be prepared from overnight cultures (18-24 hr.) that have been centrifuged at 3000g for 20 min. at 10°C. The cells will be resuspended in 0.1% peptone and standardized to the nearest whole number for percent transmission at 650 ηm with a Spectronic 100

spectrophotometer (Bausch and Lomb). The inoculum will be plated out via spiral plating using ten fold dilutions in 0.1% peptone.

5.3 In addition to the *in vitro* grown microorganisms, pooled stimulated saliva will also be used as a source of wild-type oral organisms. Stimulated saliva will be collected from a population of 6-8 healthy adult volunteers at approximately the same time (8-9 a.m.) each day. No special screening provisions will be applied for this study other than to exclude subjects who are on antibiotic therapy. The saliva donors will be asked to refrain from oral hygiene, food or drink the morning of collection. The donors will rinse their mouths with water, wait approximately five minutes, and then start chewing clean Parafilm to stimulate saliva, expectorating into a sterile plastic test tube as necessary. Saliva will be held on ice until just before use. Saliva samples will be pooled with vigorous stirring prior to exposure of the saliva to the mouthrinse samples.

6 **Kill Kinetics Assay:**

Since antimicrobial agents in the mouth are subject to inhibition by components of saliva, gingival crevicular fluid, and serum, the effect of biological fluids (e.g. horse serum or fetal bovine serum) upon antiseptic activity needs to be evaluated. Standardized preparations of sterile saliva are difficult to obtain, so heat inactivated (56 degrees C. for 60 minutes) sterile horse or bovine serum will be used to determine the effect of biological fluids on inhibition. Two mL of each organism culture will be mixed with 2 mL of sterile serum just prior to performing the kill kinetics assay. In the case of pooled saliva, no admixture with serum will be necessary.

The assay will start with the addition of one (1) mL of inoculum-serum mixture (or pooled saliva) to four (4) mL of product. The reaction tube will be vortexed for five (5) seconds and placed in a 37^0C water bath. After a 30 sec. exposure, 50 uL of reaction mixture will be dispensed into 4.95 mL of neutralizing broth and vortexed for five (5) seconds. The neutralized solution will be diluted 100 and 10,000 fold by serially transferring 100 uL into 9.9 mL of peptone, twice. Each dilution (10^0, 10^2, and 10^4) will be plated via spiral plating onto duplicate plates of nonselective blood agar (Schaedler). After 2-4 days the plates will be read via an automatic colony counter (ProtoCol Colony Counter [Bioscience International, Inc]) and the results recorded in a laboratory notebook as well as on a data sheet (see Appendix A).

7 **Data:**

The data will be transcribed from the data sheet (Appendix A) to a Microsoft Excel spread sheet in the following manner. (See Table #7.2). Data will be archived in a laboratory notebook as well.

7.1 Spiral Plater Counts:
The final counts will be determined by the Statistics and Data Management Department using the following formula: **CFU/mL = CFU counted x Multiplication Factor x Dilution**

7.2 Counts will be made using a ProtoCol Colony Counter (Bioscience International, Inc.). The multiplication factors are listed in Table #7.1 and are the reciprocal of the volume constant for 100 mm plates.

Multiplication constants for spiral plate sectors using ProtoCol plate reader
Table #7.1

Plate count obtained	**Multiplication factor**	**Volume Constant for 100 mm plates**
Whole plate	20	0.0500 mL
Spiral sector 4A	81.30	0.0123 mL
Spiral sector 4B	133.33	0.0075 mL
Spiral sector 4C	218.82	0.00457 mL
Spiral sector 3A	378.79	0.00264 mL
Spiral sector 3B	729.93	0.00137 mL
Spiral sector 3C	1851.85	0.00054 mL

Example of data on data sheet
Table #7.2

Day	Organism	random code #	Sector/ count	Sector/ count
1 example	1	12	3C 54	3C 55
1 example	1	2	3C 22	3C 25

8 Statistical Methodology

8.1 Efficacy Variables

The efficacy variables are counts of *Fusobacterium nucleatum*, *Prevotella intermedia*, *Actinomyces viscosus*, *Streptococcus sanguis*, *Streptococcus mutans*, *Candida albicans*, and salivary microorganisms. All counts will be log (base 10) transformed prior to analysis. Plate counts of 0 will be transformed to the midpoint of the threshold interval prior to the log transformation.

8.2 Sample Size

The sample size of 10 per treatment group and organism provides approximately 80 percent power to detect a difference of 0.3 log CFUs. These calculations are based on a standard deviation of 0.62 log CFUs, estimated from study #946-98-6.

8.3 Statistical Analysis

The data from this trial will be managed and analyzed by the Statistics and Data Management Department at the Warner-Lambert consumer Products Research & Development Division.

For each organism, summary statistics (mean, standard deviation, minimum and maximum) will be computed by treatment group by day and over all days.

The assumption of normally distributed errors will be assessed using the Shapiro-Wilk test.

The primary research questions are:

1. Does FreshBurst Listerine with Fluoride (W2194-471) provide significant bactericidal activity relative to the negative control (W2194-221P)?

2. Does FreshBurst Listerine with Fluoride (W2194-471) provide bactericidal activity that is at least as good as that of Listerine Antiseptic (W2194-377)?

The secondary research question is:

1. Is the study validated?

Results will be evaluated as follows:

- The Listerine with fluoride test rinse (W2194-471) will be considered significantly bactericidal for an organism if, for that organism, the mean for the test rinse is at least 3 logs lower than the mean for the negative control (W2194-221P).

- The Listerine with fluoride test rinse (W2194-471) will be considered different from the Listerine Antiseptic positive control for an organism if, for that organism, the mean for the test rinse is at least 0.25 logs higher[1] than the mean for the positive control (W2194-377).

- The study will be considered validated if, for all organisms tested, the respective means for the Listerine Antiseptic positive control are at least 3 logs lower than those for the negative control (W2194-221P).

[1] Official Methods of Analysis. Association of Official Analytical Chemists (1990). Germicidal and Detergent Sanitizing Action of Disinfectants. Final Action. Vol 1 (15th edition), pp.138-140.

Appendix A

Data Sheet

Test Date_____ Date Read_____

Day	Organism	Code #	Dilution Factor	Sector	Count	Sector	Count

Read by_____

Witness_____

24
00

FedEx. USA Airbill

FedEx Tracking Number 8210 0481 1987

SPH32

Form I.D. No. 0215

Recipient's Copy

RECIPIENT: PEEL HERE

1 From This portion can be removed for Recipient's records.

Date 5/15/00 FedEx Tracking Number 821004811987

Sender's Name M.Grilk for P. Okarma Phone

Company WARNER-LAMBERT CO

Address 170 TABOR BLDG 3 (pre-mtg) Dept./Floor/Suite/Room

City MORRIS PLAINS State NJ ZIP 07950

2 Your Internal Billing Reference N007 80 340 009 933

3 To

Recipient's Name Dockets Management Branch (HFA-305) Phone

Div. of Management Systems and Policy/ FDA

Company

5630 Fishers Lane, RM 1061

Address

To "HOLD" at FedEx location, print FedEx address. We cannot deliver to P.O. boxes or P.O. ZIP codes.

Dept./Floor/Suite/Room

City Rockville State MD ZIP 20852

8210 0481 1987

0137813254

4a Express Package Service *Packages up to 150 lbs.* Delivery commitment may be later in some areas.

- ☐ FedEx Priority Overnight — Next business morning
- ☒ FedEx Standard Overnight — Next business afternoon
- ☐ FedEx First Overnight — Earliest next business morning delivery to select locations
- ☐ FedEx 2Day* — Second business day
- ☐ FedEx Express Saver* — Third business day

* FedEx Envelope/Letter Rate not available. Minimum charge: One-pound rate

4b Express Freight Service *Packages over 150 lbs.* Delivery commitment may be later in some areas.

- ☐ FedEx 1Day Freight* — Next business day
- ☐ FedEx 2Day Freight — Second business day
- ☐ FedEx 3Day Freight — Third business day

* Call for Confirmation:

5 Packaging * Declared value limit $500

- ☐ FedEx Envelope/Letter*
- ☐ FedEx Pak*
- ☒ Other Pkg. — Includes FedEx Box, FedEx Tube, and customer pkg.

6 Special Handling Include FedEx address in Section 3.

- ☐ SATURDAY Delivery — Available for FedEx Priority Overnight and FedEx 2Day to select ZIP codes
- ☐ SUNDAY Delivery — Available for FedEx Priority Overnight to select ZIP codes
- ☐ HOLD Weekday at FedEx Location — Not available with FedEx First Overnight
- ☐ HOLD Saturday at FedEx Location — Available for FedEx Priority Overnight and FedEx 2Day to select locations

Does this shipment contain dangerous goods? One box must be checked.

- ☒ No
- ☐ Yes — As per attached Shipper's Declaration
- ☐ Yes — Shipper's Declaration not required
- ☐ Dry Ice — Dry Ice, 9, UN 1845 ____ x ____ kg

Dangerous Goods cannot be shipped in FedEx packaging. ☐ Cargo Aircraft Only

7 Payment *Bill to:* Enter FedEx Acct. No. or Credit Card No. below. ☐ Obtain Recip. Acct. No.

- ☒ Sender — Acct. No. in Section 1 will be billed.
- ☐ Recipient
- ☐ Third Party
- ☑ Credit Card
- ☐ Cash/Check

Total Packages	Total Weight		Total Charges
1	16		Credit Card Auth.

†Our liability is limited to $100 unless you declare a higher value. See the *FedEx Service Guide* for details.

8 Release Signature *Sign to authorize delivery without obtaining signature.*

By signing you authorize us to deliver this shipment without obtaining a signature and agree to indemnify and hold us harmless from any resulting claims.

Questions? Call 1·800·Go·FedEx® (800-463-3339)

Visit our Web site at www.fedex.com

SRS 100•Rev. Date 8/99•Part #154813S•©1994–99 FedEx•PRINTED IN U.S.A.

359